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*APPLICATION*

*FOR*

*UNITED STATES*

*LETTERS PATENT*

*SPECIFICATION*

(For Attorney Docket No. MTP-023)

TO ALL WHOM IT MAY CONCERN:

Be it known that we, Susan K. Keese, Robert Obar and Ying-Jye Wu, citizens of the United States of America, residing at of P.O. Box 692, 87 Oak Hill Road, Harvard, MA, 19 Lewis Avenue, Walpole, MA; and 71 Flanagan Drive, Framingham, MA, respectively in the United States of America, have invented new and useful improvements in

"METHODS AND COMPOSITIONS FOR THE DETECTION OF CERVICAL CANCER"

of which the following is a specification.

**METHODS AND COMPOSITIONS FOR THE  
DETECTION OF CERVICAL CANCER**

**Field of the Invention.**

The present invention relates generally to methods and compositions for the detection of cervical cancer. More specifically, the present invention relates to cervical cancer-associated proteins which act as cellular markers useful (i) in detecting cervical cancer, and (ii) as molecular targets for cervical cancer therapy.

**Background of the Invention.**

Cancer of the uterine cervix is one of the most common malignancies in women and remains a significant public health problem throughout the world. In the United States alone, invasive cervical cancer accounts for approximately 19% of all gynecological cancers (Miller *et al.* (1993) in "Surveillance Epidemiology, and End Results Program cancer Statistics Review: 1973-1990", NIH Pub. No. 93-2789, Bethesda, MD: National Cancer Institute). In 1996, it is estimated that there will be 14,700 newly diagnosed cases and 4900 deaths attributed to this disease (American Cancer Society, Cancer Facts & Figures 1996, Atlanta, GA: American Cancer Society, 1996). In many developing countries, where mass screening programs are not widely available, the clinical problem is more serious. Worldwide, the number of new cases is estimated to be 471,000 with a 4 year survival rate of 40% (Munoz *et al.* (1989) "Epidemiology of Cervical Cancer" in "Human Papillomavirus", New York, Oxford Press, pp 9-39; and National Institutes of Health, Consensus Development Conference Statement on Cervical Cancer, April 1-3, 1996).

The precursor to cervical cancer is dysplasia, also known in the art as cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL) (Brinton *et al.* (1992) “*Epidemiology of Cervical Cancer: Overview*” in “*The Epidemiology of Cervical Cancer and Human Papillomavirus*”, Lyon, France: International Agency for Research on Cancer; and  
5   Tabbara *et al.* (1992) “*The Bethesda classification for squamous intraepithelial lesions: histologic, cytologic and viral correlates*”, *Obstet. Gynecol.* 79: 338-346). While it is not understood how normal cells become transformed, the concept of a continuous spectrum of histopathological change from normal, stratified epithelium through CIN to invasive cancer has been widely accepted for many years (*see, for example*, Mitchell *et al.* (1994) “*The natural*  
10   *history of cervical intraepithelial neoplasia: an argument of intermediate endpoint biomarkers*”, *Cancer Epidemiol. Biomark. Prev.* 3: 619-626). A large body of epidemiological and molecular biological evidence has been gathered that establishes human papillomavirus (HPV) infection as a causative factor in cervical cancer (Munoz *et al.* (1992) in “*The Epidemiology of Human Papillomavirus and Cervical Cancer*”, IRAC publication no. 119, Lyon France: Int. Agency for  
15   Research on Cancer, pp 251-261). HPV is found in 85% or more of squamous cell invasive lesions, which represent the most common histologic type seen in cervical carcinoma (Cox *et al.* (1995) *Baillierre’s Clin. Obstet Gynaecol.* 91-37). Additional cofactors include, for example, oncogenes activated by point mutations, and chromosomal translocations or deletions (Spandidos  
20   *et al.* (1989) *J. Pathol.* 157: 1-10).

Cytological examination of Papanicolaou-stained cervical smears (also referred to as Pap smears) currently is the method of choice for detecting cervical cancer. Despite the historical success of this test, concerns have arisen regarding its ability to predict reliably the behavior of same preinvasive lesions (Ostor *et al.* (1993) *Int. J. Gynecol. Pathol.* 12: 186-192; and Genest *et al.* (1993) *Human Pathol.* 24: 730-736). The identification of a cervical cancer-associated tumor  
25   marker for reliably detecting early onset of cervical cancer and/or providing early prognostic information will greatly aid the management of cervical cancer.

All eukaryotic cells have a nucleus containing DNA, or chromatin, which is organized by  
30   an internal protein scaffolding known as the nuclear matrix (NM). The nuclear matrix was first

described in 1974 by Berezney *et al.* (Berezney *et al.* (1974) *Biochem. Biophys. Res. Commun.*, 60: 1410-1417). Penman *et al.* describe a method for selectively extracting insoluble interior nuclear matrix proteins and their associated nucleic acids from cells and determining the particular cell type by analyzing the proteins by two-dimensional gel electrophoresis (see for example, U.S. Pat. Nos. 4,882,268, issued 11/21/89, and 4,885,236, issued 12/5/89, the disclosures of which are incorporated herein by reference).

The nuclear matrix is believed to be involved in a wide variety of nuclear functions fundamental to the control of gene expression. For a general review see, for example, Fey *et al.* (1991) *Crit. Rev. Euk. Gene Express.* 1: 127-143. Tissue-specific nuclear matrix proteins have been identified in the rat, mouse and human. Fey *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 85: 121-125; Stuurman *et al.* (1990) *J. Biol. Chem.* 265: 5460-5465; and Getzenberg *et al.* (1990) *Mol. Endocrinol.* 4: 1336-1342. Changes in the presence or absence of specific nuclear matrix proteins have been associated with cellular transformation and differentiation (Bidwell *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90: 3162-3166; Brancolini *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88: 6936-6940; and Greenfield *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88: 11217-11221).

Several recent studies using similar methodology have identified tumor-specific nuclear matrix proteins in cancers of the prostate (Partin *et al.* (1993) *Cancer Res.* 53: 744-746), breast (Khanuja *et al.* (1993) *Cancer Res.* 53: 3394-3398), colon cancer (Keese *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91: 1913-1916), bone (Bidwell *et al.* (1994) *Cancer Res.* 54: 28-32), bladder (Getzenberg *et al.* (1996) *Cancer Res.* 56: 690-694) and the larynx (Donat *et al.* (1996) *Otolaryngol. Head Neck Surg.* 114: 387-393). Molecular characterization of the specific nuclear matrix proteins, however, remains poorly defined, due to the low abundance of these proteins in the cell and their generally insoluble character.

There is, however, a need in the art for specific, reliable markers that are expressed differentially in normal and cancerous cervical tissue and that may be useful in the detecting cervical cancer or in the prediction of its onset. Accordingly, it is an object of this invention to

provide cervical cancer-associated molecules which are useful as markers for the early and/or rapid detection of cervical cancers in an individual. It is another object of this invention to provide methods for detecting cervical cancers in an individual. It is another object of the invention to provide methods and compositions for treating cervical cancers in an individual and  
5 for monitoring the efficacy of such a treatment in the individual.

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### Summary of the Invention.

The invention provides a variety of methods and compositions for detecting and/or prognosing cervical cancer in a tissue or body fluid sample of an individual. The invention is based, in part, upon the discovery of cervical cancer-associated proteins which are present at detectable levels in cervical cancer cells, but which are not detectable in normal cervical cells, as determined by two-dimensional gel electrophoresis.

In one aspect, the invention provides a method for detecting cervical cancer in a human. The method comprises the step of detecting the presence of a cervical cancer-associated protein in a tissue or body fluid sample of the human thereby to indicate the presence of a cervical cancer or a precursor of a cervical cancer. The cervical cancer-associated protein is characterized as having a molecular weight of from about 44,900 Daltons to about 69,400 Daltons, as determined by standard polyacrylamide gel electrophoresis techniques and an isoelectric point of from about 5.1 to about 6.6 as determined by standard isoelectric focusing techniques. In addition, the cervical cancer-associated protein is further characterized as being a non-chromatin protein which is detectable at a higher level in a human cervical cancer cell than in a normal human cervical cell, as determined by two-dimensional gel electrophoresis. It is contemplated, however, that the accuracy and/or reliability of the method may be further enhanced by detecting the presence of a plurality of cervical cancer-associated proteins in the preselected tissue or body fluid sample.

As used herein, the term "cervical cancer" is understood to mean any cancer or cancerous lesion associated with cervical tissue or cervical cells and, in addition, includes precursors to cervical cancer, for example, dysplasia (also known in the art as a cervical intraepithelial neoplasia or a squamous intraepithelial lesion).

As used herein, the term "cervical cancer-associated" molecules refers to molecules originating from and isolatable from a cervical cancer cell or cells, and substantially neither originating from nor isolatable from a normal cervical cancer cell or cells. As used herein, the

term "cervical cancer-associated protein" is understood to mean any protein which is detectable at a higher level in cervical cancer cells than in normal cervical cells, as determined by two-dimensional (2-D) gel electrophoresis. It is not necessary that the target molecule or target protein be unique to a cervical cancer cell; rather it is preferred that the target molecule or protein has a signal to noise ration high enough to discriminate between samples originating from a cervical cancer tissue or body fluid and samples originating from normal cervical tissue or body fluid.

In a preferred embodiment, methods of the invention comprise the step of detecting one or more cervical cancer (CvC) associated proteins, referred to herein as CvC-1 through CvC-5, which can be purified or co-purified using nuclear matrix protein purification methodologies, well known and thoroughly documented in the art. See, for example, Fey *et al.* (1986) *Proc. Natl. Acad. Sci, USA* 85: 121-125, the disclosure of which is incorporated herein by reference. As used herein, the term "nuclear matrix protein" is understood to mean any non-cytoskeletal, non-lamin, non-chromatin protein that (i) is isolated from mammalian cell nuclei, (ii) is resistant to solubilization from the nuclei in 0.25M ammonium sulfate, (iii) remains in solution following dialysis into physiological buffer from 8M urea and (iv) is detectable on a silver stained two-dimensional electrophoresis gel. Accordingly, one or more of the resultant cervical cancer - associated proteins may be further defined as being a nuclear matrix protein.

In a preferred embodiment, methods of the invention may comprise the step of detecting the protein CvC-1, a protein having a molecular weight of about 69,400 Daltons, as determined by polyacrylamide gel electrophoresis, and a pI of about 5.8, as determined by isoelectric focusing techniques. Alternatively, the methods of the invention may comprise the step of detecting the protein CvC-2, a protein having a molecular weight of about 53,800 Daltons, as determined by polyacrylamide gel electrophoresis, and a pI of about 5.5, as determined by isoelectric focusing techniques. Alternatively, the methods of the invention may comprise the step of detecting the protein CvC-3, a protein having a molecular weight of about 47,900 Daltons, as determined by polyacrylamide gel electrophoresis, and a pI of about 5.6, as determined by isoelectric focusing techniques. Alternatively, the methods of the invention may

comprise the step of detecting the protein CvC-4, a protein having a molecular weight of about 46,000 Daltons, as determined by polyacrylamide gel electrophoresis, and a pI of about 5.1, as determined by isoelectric focusing techniques. Alternatively, the methods of the invention may comprise the step of detecting the protein CvC-5, a protein having a molecular weight of about 44,900 Daltons, as determined by polyacrylamide gel electrophoresis, and a pI of about 6.6, as determined by isoelectric focusing techniques.

In another preferred embodiment, the methods of the invention may comprise the step of detecting a cervical cancer-associated protein which comprises a continuous amino acid sequence selected from the group consisting of: SEQ ID NO.: 1; SEQ ID NO.: 2; SEQ ID NO.: 3; SEQ ID NO.: 4; SEQ ID NO.: 5; SEQ ID NO.: 6; SEQ ID NO.: 7; SEQ ID NO.: 8; and SEQ ID NO.: 9. Alternatively, the method of the invention may comprise the step of detecting a cervical cancer-associated protein having the amino acid sequence set forth in SEQ ID NO.: 10, commonly referred to in the art as IEF SSP 9502. See, for example, Honore *et al.* (1994) *Gene* **151**: 291-296, the disclosure of which is incorporated herein by reference.

In another preferred embodiment, the methods of the invention may comprise the step of detecting a cervical cancer-associated protein which comprises a continuous amino acid sequence selected from the group consisting of: SEQ ID NO.: 11; SEQ ID NO.: 12; SEQ ID NO.: 13; SEQ ID NO.: 14; SEQ ID NO.: 15; SEQ ID NO.: 16; and SEQ ID NO.: 17. Alternatively, the method of the invention may comprise the step of detecting a cervical cancer-associated protein having the amino acid sequence set forth in SEQ ID NO.: 18, and commonly referred to in the art as Cytokeratin 17. See, for example, Troyanovsky *et al.* (1992) *J. Biol. Biol.* **59**: 127-137, the disclosure of which is incorporated herein by reference.

In another preferred embodiment, the methods of the invention may comprise the step of detecting a cervical cancer-associated protein which comprises a continuous amino acid sequence selected from the group consisting of: SEQ ID NO.: 19; SEQ ID NO.: 20; SEQ ID NO.: 21; SEQ ID NO.: 22; SEQ ID NO.: 23; SEQ ID NO.: 24; and SEQ ID NO.: 25. Alternatively, the method of the invention may comprise the step of detecting a cervical cancer-associated protein having



the amino acid sequence set forth in SEQ ID NO.: 26, commonly referred to in the art as TDP-43. See, for example, Ou *et al.* (1995) *J. Virol.* 69: 3584-3596, the disclosure of which is incorporated herein by reference.

5 In another preferred embodiment, the methods of the invention may comprise the step of detecting a cervical cancer-associated protein which comprises a continuous amino acid sequence selected from the group consisting of: SEQ ID NO.: 27; SEQ ID NO.: 28; SEQ ID NO.: 29; SEQ ID NO.: 30; SEQ ID NO.: 31; SEQ ID NO.: 32; and SEQ ID NO.: 33. Alternatively, the method of the invention may comprise the step of detecting a cervical cancer-associated protein having  
10 the amino acid sequence set forth in SEQ ID NO.: 34, commonly referred to in the art as Nup358. See, for example, Wu *et al.* (1995) *J. Biol. Chem.* 270: 14209-14213, the disclosure of which is incorporated herein by reference.

In another preferred embodiment, the methods of the invention may comprise the step of  
15 detecting a cervical cancer-associated protein which comprises a continuous amino acid sequence selected from the group consisting of: SEQ ID NO.: 35; SEQ ID NO.: 36; SEQ ID NO.: 37; SEQ ID NO.: 38; SEQ ID NO.: 39; SEQ ID NO.: 40; SEQ ID NO.: 41; SEQ ID NO.: 42; SEQ ID NO.: 43; SEQ ID NO.: 44; and SEQ ID NO.: 45. Alternatively, the method of the invention may comprise the step of detecting a cervical cancer-associated protein having the amino acid  
20 sequence set forth in SEQ ID NO.: 46, commonly referred to in the art as lamin A. See, for example, Fisher *et al.* (1986) *Proc. Natl. Acad. Sci. USA.* 83: 6450-6454, the disclosure of which is incorporated herein by reference.

The methods of the invention may be performed on any relevant tissue or body fluid  
25 sample. For example, methods of the invention may be performed on cervical tissue, more preferably cervical biopsy tissue, and most preferably on Pap smears. Alternatively, the methods of the invention may be performed on a human body fluid sample selected from the group consisting of: blood; serum; plasma; fecal matter; urine; vaginal secretion; spinal fluid; saliva; ascitic fluid; peritoneal fluid; sputum; and breast exudate. It is contemplated, however, that the

methods of the invention also may be useful in assays for metastasized cervical cancer cells in other tissue or body fluid samples.

Marker proteins associated with a cervical cancer in a tissue or body fluid sample may be detected using any of a number of assay methods available in the art. In one embodiment, for example, the marker cervical cancer-associated protein may be reacted with a labeled binding moiety capable of specifically binding to the marker protein thereby to produce a labeled complex of the binding moiety and the marker protein. The labeled complex thereafter may be detected, using conventional methodologies well known in the art. Detection of the presence of the labeled complex may provide an indication of the presence of the cervical cancer cells or pre-cancerous cells in the individual being tested. As used herein, the term "binding moiety" is understood to mean any binding partner capable of specifically binding to a cervical cancer-associated protein with a binding affinity greater than about  $10^5 \text{ M}^{-1}$ . As used herein the terms "specifically binding", "specifically bound" and "binds specifically" refer to a binding interaction with a binding affinity of greater than about  $10^5 \text{ M}^{-1}$ . As used herein, the binding moiety is labeled with a detectable moiety, for example, a radioactive, fluoroscopic, spectroscopic, or enzymatic label, using techniques well known in the art.

It is appreciated that, binding moieties which interact and bind specifically with the target protein, may be designed using conventional methods well known in the art. In the invention, the binding moiety can be an antibody, for example, a monoclonal or a polyclonal antibody. Monoclonal antibodies are preferred. It is contemplated, however, that other useful binding moieties useful in the practice of the instant invention may include, for example, biosynthetic antibody binding sites, also referred to in the art as BABS or sFv's, and antibody fragments, for example, Fv, Fab, Fab' and (Fab')<sub>2</sub> fragments. Procedures for preparing, testing, and labeling BABS and antibody fragments are well known in the art, and so are not discussed in detail herein.

In another embodiment, one or more marker proteins in a sample may be detected by first isolating the proteins from the sample, and then separating the proteins by two-dimensional gel electrophoresis to produce a characteristic two-dimensional gel electrophoresis pattern. The gel electrophoresis pattern then may be compared with a standard, for example, a standard gel pattern obtained from a data base of gel electrophoresis patterns. Thus, in another embodiment, the invention provides electrophoresis gel patterns or electropherograms of cervical cancer-associated proteins which are useful in detecting a cervical cancer in an individual.

The cervical cancer-associated proteins of the invention can be purified or co-purified from cervical cancer cells using nuclear matrix protein isolation procedures, such as those disclosed in U.S. Patent No. 4,885,236 and U.S. Patent No. 4,882,268, the disclosures of which are incorporated herein. Alternatively, the marker proteins, once identified and characterized may be isolated from the sample by any of a range of protein purification protocols well known to those skilled in the art, such as affinity chromatography, to yield isolated proteins. As used herein, the term "isolated" is understood to mean substantially free of undesired, contaminating proteinaceous material.

Furthermore, the skilled artisan may produce nucleic acid sequences encoding the entire isolated marker protein, or fragments thereof, using methods currently available in the art (see, for example, Maniatis *et al.*, eds. (1989) "*Molecular Cloning: A Laboratory Manual*," Cold Spring Harbor Press). For example, an isolated cervical cancer-associated protein may be sequenced using conventional peptide sequencing protocols, and then oligonucleotide hybridization probes designed for screening a cDNA library. The cDNA library then may be screened with the resultant oligonucleotide to isolate full or partial length cDNA sequences which encode the isolated protein.

Furthermore, the skilled artisan, using the methodologies described in U.S. Patent Nos. 4,885,236 and 4,882,268 may isolate from a cell sample a nucleic acid molecule having a sequence capable of recognizing and being specifically bound by a cervical cancer-associated protein. In such a procedure, the soluble proteins are separated from the nucleus and

cytoskeleton by extracting mammalian cells with a non-ionic detergent solution at physiological pH and ionic strength. The insoluble protein and nucleic acids then are digested with DNAase and then eluted with a buffered ammonium sulfate solution to yield a nucleic acid molecule capable of recognizing and being specifically bound by a cervical cancer-associated protein. Any remaining proteins then are separated from the target nucleic acid molecule.

Detection of the aforementioned nucleic acid molecules thus can serve as an indicator of the presence of cervical cancer and/or metastasized cervical cancer in an individual. Accordingly, in another aspect, the invention provides another method for detecting cervical cancer in a human. The method comprises the step of detecting the presence of a nucleic acid molecule in a tissue or body fluid sample thereby to indicate the presence of a cervical carcinoma in the individual. The nucleic acid molecule is selected from the group consisting of (i) a nucleic acid molecule comprising a sequence capable of recognizing and being specifically bound by a cervical cancer-associated protein, and (ii) a nucleic acid molecule comprising a sequence encoding a cervical cancer-associated protein. As defined herein, the cervical cancer-associated protein is characterized as being selected from the group consisting of (i) a protein having a molecular weight of about 69,400 Daltons and an isoelectric point of about 5.8; (ii) a protein having a molecular weight of about 53,800 Daltons and an isoelectric point of about 5.5; (iii) a protein having a molecular weight of about 47,900 Daltons and an isoelectric point of about 5.6; (iv) a protein having a molecular weight of about 46,000 Daltons, and an isoelectric point of about 5.1; and (v) a protein having a molecular weight of about 44,900 Daltons and an isoelectric point of about 6.6, wherein in each example, the molecular weight is determined by standard polyacrylamide gel electrophoresis techniques and the isoelectric point is determined by standard isoelectric focusing techniques, and wherein the cervical cancer-associated protein is further characterized as being a non-chromatin protein which is detectable at a higher level in a human cervical cancer cell than in a normal human cervical cell, as determined by two-dimensional gel electrophoresis.

A target nucleic acid molecule in a sample may be detected, for example, by Northern blot analysis by reacting the sample with a labeled hybridization probe, for example, a <sup>32</sup>P labeled oligonucleotide probe, capable of hybridizing specifically with at least a portion of the nucleic acid molecule encoding the marker protein. Detection of a nucleic acid molecule either encoding  
5 a cervical cancer-associated protein or capable of being specifically bound by a cervical cancer-associated protein, thus can serve as an indicator of the presence of a cervical cancer in the individual being tested.

In another aspect, the invention provides a kit for detecting the presence of cervical cancer  
10 or for evaluating the efficacy of a therapeutic treatment of a cervical cancer. Such kits may comprise, in combination, (i) a receptacle for receiving a human tissue or body fluid sample from the individual, (ii) a binding partner which binds specifically either to an epitope on a marker cervical cancer-associated protein or a nucleic acid sequence encoding at least a portion of the  
15 marker cervical cancer-associated protein, (iii) means for detecting the binding of the binding partner with either the cervical cancer-associated protein or the nucleic acid sequence encoding at least a portion of the cervical cancer-associated protein, and (iv) a reference sample.

In one embodiment of the kit, the binding moiety binds specifically to a cervical cancer-associated protein selected from the group of proteins further defined as having: a molecular  
20 weight of about 69,400 Daltons and an isoelectric point of about 5.8; a molecular weight of about 53,800 Daltons and an isoelectric point of about 5.5; a molecular weight of about 47,900 Daltons and an isoelectric point of about 5.6; a molecular weight of about 46,000 Daltons and an isoelectric point of about 5.1, or a molecular weight of about 44,900 Daltons and an isoelectric point of about 6.6, wherein the molecular weight is determined by conventional polyacrylamide  
25 gel electrophoresis methodologies, and the isoelectric point is determined by conventional isoelectric focusing methodologies.

In another embodiment of the kit, the reference sample may comprise a negative and/or positive control. The negative control being indicative of a normal cervical cell type and the  
30 positive control being indicative of cervical cancer.

In another aspect, the invention provides a method for treating cervical cancer. The method comprises administering to a patient with cervical cancer, a therapeutically-effective amount of a compound, preferably an antibody, and most preferably a monoclonal antibody, which binds specifically to a target cervical cancer-associated protein thereby to inactivate the protein. The target protein being characterized as having a molecular weight of from about 44,900 Daltons to about 69,400 Daltons, as determined by standard polyacrylamide gel electrophoresis techniques and an isoelectric point of from about 5.1 to about 6.6, as determined by standard isoelectric focusing techniques, and wherein the target protein is further characterized as being a non-chromatin protein which is detectable at a higher level in a human cervical cancer cell than in a normal human cervical cell, as determined by two-dimensional gel electrophoresis. Similarly, it is contemplated that the compound may comprise a small molecule, for example, as small organic molecule, which inhibits or reduces the biological activity of the target cervical cancer-associated protein.

In another aspect, the invention provides another method for treating cervical cancer. The method comprises the step of administering to a patient diagnosed as having cervical cancer, a therapeutically-effective amount of a compound which reduces *in vivo* the expression of a target cervical cancer-associated protein thereby to reduce *in vivo* the expression of the target protein.

In a preferred embodiment, the compound is a nucleobase containing sequence, such as, an anti-sense nucleic acid sequence or anti-sense peptide nucleic acid (PNA) molecule, complementary to a nucleic acid sequence encoding at least a portion of the target protein. After administration, the anti-sense nucleic acid sequence or anti-sense PNA molecule binds to the nucleic acid sequences encoding, at least in part, the target protein thereby to reduce *in vivo* expression of the target cervical cancer-associated protein.

Thus, the invention provides a wide range of methods and compositions for detecting and treating cervical cancer in an individual. Specifically, the invention provides cervical cancer-associated proteins, which permit specific and early, preferably before metastases occur, detection of cervical cancer in an individual. In addition, the invention provides kits useful in the



**Brief Description of the Drawings.**

Figure 1a is a high resolution two-dimensional gel electrophoresis pattern of nuclear matrix proteins isolated from a cervical cancer tissue sample. Tumor-associated proteins encircled and marked with reference numbers 1-5 correspond to proteins CvC-1 to CvC-5, listed in Table 2.

Figure 1b is a high resolution two-dimensional gel electrophoresis pattern of nuclear matrix proteins isolated from a normal cervical tissue sample. As a reference, the relative positions corresponding to the CvC-1 to CvC-5 proteins of Figure 1a are encircled and marked with reference numbers 1-5.

Figure 2a is a high resolution two-dimensional gel electrophoresis pattern of nuclear matrix proteins isolated from the cervical carcinoma-derived cell line C33A. Tumor-associated proteins CvC-2 and CvC-5 are encircled and marked with reference numbers 2 and 5.

Figure 2b is a high resolution two-dimensional gel electrophoresis pattern of nuclear matrix proteins isolated from CaSki cells. Tumor-associated proteins CvC-1 and CvC-3 are encircled and marked with reference numbers 1 and 3.

For each of the above figures, molecular weight standards are indicated on the ordinate axes ( $M_r \times 10^3$ ) and isoelectric points are shown on the abscissae.



### Detailed Description of the Invention.

The present invention provides methods and compositions for the detection and treatment of cervical cancer. The invention is based, in part, upon the discovery of cervical cancer-associated proteins which generally are present at detectably higher levels in cancerous cervical cells than in normal cervical cells, as determined by two-dimensional gel electrophoresis.

The cervical cancer-associated proteins may act as marker proteins useful in the detection of cervical cancer or as target proteins for therapy of cervical cancer. For example, it is contemplated that, the marker proteins and binding moieties, for example, antibodies that bind to the marker proteins or nucleic acid probes which hybridize to nucleic acid sequences encoding the marker proteins, may be used to detect the presence of cervical cancer in an individual.

Furthermore, it is contemplated that, the skilled artisan may produce novel therapeutics for treating cervical cancer which include, for example: antibodies which can be administered to an individual that bind to and reduce or eliminate the biological activity of the target protein *in vivo*; nucleic acid or peptide nucleic acid sequences which hybridize with genes or gene transcripts encoding the target proteins thereby to reduce expression of the target proteins *in vivo*; or small molecules, for example, organic molecules which interact with the target proteins or other cellular moieties, for example, receptors for the target proteins, thereby to reduce or eliminate biological activity of the target proteins.

Set forth below are methods for isolating cervical cancer-associated proteins, methods for detecting cervical cancer using cervical cancer-associated proteins as markers, and methods for treating individuals afflicted with cervical cancer using cervical cancer-associated proteins as targets for cancer therapy.

#### 1. Identification and Purification of Cervical Cancer-associated Proteins.

Marker proteins of the invention, as disclosed herein are identified by (i) isolating proteins from normal cervical tissue and from cervical cancer tissue using a nuclear matrix purification

protocol, such as those described generally in U.S. Pat. Nos. 4,882,268 and 4,885,236, or Fey et al. (1986) *supra* (ii) fractionating the resulting nuclear matrix protein preparations by 2-D gel electrophoresis, (iii) visualizing the resulting protein patterns, for example, by silver staining, and (iv) identifying polypeptide spots on the resulting 2-D gel electropherograms which generally are detectable in samples isolated from cervical cancer cells but not detectable in samples isolated from normal cervical cells.

Marker proteins associated with cervical cancer tissue were isolated as described herein using a modification of the method of Fey et al. (Fey et al. (1986) *supra*). Briefly, cervical cancer tissue is minced into small (1 mm<sup>3</sup>) pieces and homogenized with a Teflon pestle on ice and treated with a buffered solution containing 0.5% Triton-X-100, vanadyl riboside complex plus a protease inhibitor cocktail (phenylmethyl sulfonyl fluoride, aprotinin, and leupeptin) to remove lipids and soluble proteins. Tumor cells from cell lines can be harvested by trypsinization and treated in the same way as for homogenized tumor tissue. Stromal aggregates are removed by filtering the homogenate through a 250 micron nylon screen followed by a centrifugation step.

Soluble cytoskeletal proteins are removed by incubating the pellet in an extraction buffer containing 250 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5% Triton-X-100, vanadyl riboside complex plus a protease inhibitor cocktail for 10 minutes on ice followed by centrifugation. Chromatin is removed by incubating the pellet in DNAase I in a buffered solution containing a protease inhibitor cocktail for 45 minutes at 25°C.

The remaining pellet fraction, containing the target proteins and intermediate filaments, is solubilized in a disassembly buffer containing 8 M urea, protease inhibitor cocktail plus 1% 2-mercaptoethanol. Insoluble contaminants, primarily carbohydrates and extracellular matrix, are removed by ultracentrifugation. Intermediate filaments are allowed to reassemble upon removal of urea by dialysis in assembly buffer containing protease inhibitor cocktail and removed by ultracentrifugation, leaving the target proteins in the supernatant fraction. Protein concentration

can be determined by the Coomassie Plus Protein Assay Kit (Pierce Chemicals, Rockford, IL) using a bovine gamma globulin standard. Proteins are immediately precipitated in 80% ethanol and stored at -80°C until use.

5 It is contemplated that, after identification, the resulting cervical cancer-associated proteins may be isolated by preparing a nuclear matrix protein preparation, such as the one described above, electrophoresing the resulting proteins on a 2-D gel, and after some means of visualization, isolating the protein of interest from the resulting 2-D gel. Alternatively, it is contemplated that the marker protein, once identified, can be isolated, using standard protein  
10 purification methodologies well known to those of ordinary skill in the art, such as affinity chromatography, to yield substantially pure marker proteins. As used herein, the term "substantially pure" is understood to mean at least 80% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

## 15 2. Detection of Cervical Cancer.

Once cervical cancer-associated proteins have been identified, they may be used as markers to determine whether an individual has cervical cancer and/or cervical dysplasia, and if so, suitable detection methods can be used to monitor the status of the disease.

20 Using the marker proteins, the skilled artisan can produce a variety of detection methods for detecting cervical cancer in a human. The methods, typically comprise the steps of detecting, by some means, the presence of one or more cervical cancer-associated proteins in a tissue or body fluid sample of the human. The accuracy and/or reliability of the method for detecting cervical  
25 cancer in a human may be further enhanced by detecting the presence of a plurality of cervical cancer-associated proteins in a preselected tissue or body fluid sample. The detection step may comprise one or more of the protocols described hereinbelow.

## 2.A. Protein Detection Methods.

The marker protein in a sample may be reacted with a binding moiety capable of specifically binding the marker protein. The binding moiety may comprise, for example, a member of a ligand-receptor pair, i.e., a pair of molecules capable of having a specific binding interaction. The binding moiety may comprise, for example, a member of a specific binding pair, such as antibody-antigen, enzyme-substrate, nucleic acid-nucleic acid, protein-nucleic acid, protein-protein, or other specific binding pair known in the art. Binding proteins may be designed which have enhanced affinity for a target protein. Optionally, the binding moiety may be linked with a detectable label, such as an enzymatic, fluorescent, radioactive, phosphorescent or colored particle label. The labeled complex may be detected, e.g., visually or with the aid of a spectrophotometer or other detector.

The marker proteins also may be detected using gel electrophoresis techniques available in the art. In two-dimensional gel electrophoresis, the proteins are separated first in a pH gradient gel according to their isoelectric point. The resulting gel then is placed on a second polyacrylamide gel, and the proteins separated according to molecular weight (see, for example, O'Farrell (1975) *J. Biol. Chem.* 250: 4007-4021).

One or more marker proteins may be detected by first isolating proteins from a sample obtained from an individual suspected of having cervical cancer, and then separating the proteins by two-dimensional gel electrophoresis to produce a characteristic two-dimensional gel electrophoresis pattern. The pattern then may be compared with a standard gel pattern produced by separating, under the same or similar conditions, proteins isolated from normal or cancer cells. The standard may be stored or obtained in an electronic database of electrophoresis patterns. The presence of a cervical cancer-associated protein in the two-dimensional gel provides an indication of the presence of a cervical cancer in the sample being tested. The detection of two or more proteins in the two-dimensional gel electrophoresis pattern further enhances the accuracy of the assay. The presence of a plurality, e.g., two to five, cervical cancer-associated proteins on the two-dimensional gel provides a strong indication of the presence of a

cervical cancer in the sample. The assay thus permits the early detection and treatment of cervical cancer.

## 2B. Immunoassay.

5

A marker cervical cancer-associated protein may also be detected using any of a wide range of immunoassay techniques available in the art. For example, the skilled artisan may employ the sandwich immunoassay format to detect cervical cancer in a body fluid sample. Alternatively, the skilled artisan may use conventional immuno-histochemical procedures for detecting the presence of the cervical cancer-associated protein in a tissue sample, for example, in a Pap smear, using one or more labeled binding proteins (See Example 5, hereinbelow).

15 In a sandwich immunoassay, two antibodies capable of binding the marker protein generally are used, e.g., one immobilized onto a solid support, and one free in solution and labeled with detectable chemical compound. Examples of chemical labels that may be used for the second antibody include radioisotopes, fluorescent compounds, and enzymes or other molecules which generate colored or electrochemically active products when exposed to a reactant or enzyme substrate. When a sample containing the marker protein is placed in this system, the marker protein binds to both the immobilized antibody and the labeled antibody, to form a "sandwich" immune complex on the support's surface. The complexed protein is detected by washing away non-bound sample components and excess labeled antibody, and measuring the amount of labeled antibody complexed to protein on the support's surface.

25 Both the sandwich immunoassay and the tissue immunohistochemical procedure are highly specific and very sensitive, provided that labels with good limits of detection are used. A detailed review of immunological assay design, theory and protocols can be found in numerous texts in the art, including "*Practical Immunology*", Butt, W.R., ed., (1984) Marcel Dekker, New York and "*Antibodies, A Laboratory Approach*" Harlow *et al.* eds.(1988) Cold Spring Harbor Laboratory.

30

In general, immunoassay design considerations include preparation of antibodies (e.g., monoclonal or polyclonal antibodies) having sufficiently high binding specificity for the target protein to form a complex that can be distinguished reliably from products of nonspecific interactions. As used herein, the term "antibody" is understood to mean binding proteins, for example, antibodies or other proteins comprising an immunoglobulin variable region-like binding domain, having the appropriate binding affinities and specificities for the target protein. The higher the antibody binding specificity, the lower the target protein concentration that can be detected. A preferred binding specificity is such that the binding protein has a binding affinity for the target protein of greater than about  $10^5 \text{ M}^{-1}$ , preferably greater than about  $10^7 \text{ M}^{-1}$ .

Antibodies to an isolated target cervical cancer-associated protein which are useful in assays for detecting a cervical cancer in an individual may be generated using standard immunological procedures well known and described in the art. See, for example, *Practical Immunology*, Butt, N.R., ed., Marcel Dekker, NY, 1984. Briefly, an isolated target protein is used to raise antibodies in a xenogeneic host, such as a mouse, goat or other suitable mammal.

The marker protein is combined with a suitable adjuvant capable of enhancing antibody production in the host, and injected into the host, for example, by intraperitoneal administration. Any adjuvant suitable for stimulating the host's immune response may be used. A commonly used adjuvant is Freund's complete adjuvant (an emulsion comprising killed and dried microbial cells and available from, for example, Calbiochem Corp., San Diego, or Gibco, Grand Island, NY). Where multiple antigen injections are desired, the subsequent injections comprise the antigen in combination with an incomplete adjuvant (e.g., cell-free emulsion).

Polyclonal antibodies may be isolated from the antibody-producing host by extracting serum containing antibodies to the protein of interest. Monoclonal antibodies may be produced by isolating host cells that produce the desired antibody, fusing these cells with myeloma cells using standard procedures known in the immunology art, and screening for hybrid cells (hybridomas) that react specifically with the target protein and have the desired binding affinity.

Antibody binding domains also may be produced biosynthetically and the amino acid sequence of the binding domain manipulated to enhance binding affinity with a preferred epitope on the target protein. Specific antibody methodologies are well understood and described in the literature. A more detailed description of their preparation can be found, for example, in "Practical Immunology" (1984) *supra*)

In addition, genetically engineered biosynthetic antibody binding sites, also known in the art as BABS or sFv's, may be used in the practice of the instant invention. Methods for making and using BABS comprising (i) non-covalently associated or disulfide bonded synthetic V<sub>H</sub> and V<sub>L</sub> dimers, (ii) covalently linked V<sub>H</sub>-V<sub>L</sub> single chain binding sites, (iii) individual V<sub>H</sub> or V<sub>L</sub> domains, or (iv) single chain antibody binding sites are disclosed, for example, in U.S. Patent Nos.: 5,091,513; 5,132,405; 4,704,692; and 4,946,778, the disclosures of which are incorporated herein by reference. Furthermore, BABS having requisite specificity for the cervical cancer-associated proteins can be derived by phage antibody cloning from combinatorial gene libraries (see, for example, Clackson *et al.* (1991) *Nature* 352: 624-628). Briefly, a library of phage each of which express on their coat surface, BABS having immunoglobulin variable regions encoded by variable region gene sequences derived from mice pre-immunized with isolated cervical cancer-associated proteins, or fragments thereof, are screened for binding activity against immobilized cervical cancer-associated protein. Phage which bind to the immobilized cervical cancer-associated proteins are harvested and the gene encoding the BABS sequenced. The resulting nucleic acid sequences encoding the BABS of interest then may be expressed in conventional expression systems to produce the BABS protein.

The isolated cervical cancer-associated protein also may be used for the development of diagnostic and other tissue evaluating kits and assays to monitor the level of the proteins in a tissue or fluid sample. For example, the kit may include antibodies or other specific binding proteins which bind specifically with the cervical cancer-associated proteins and which permit

the presence and/or concentration of the cervical cancer-associated proteins to be detected and/or quantitated in a tissue or fluid sample.

Suitable kits for detecting cervical cancer-associated proteins are contemplated to include, e.g., a receptacle or other means for capturing a sample to be evaluated, and means for detecting the presence and/or quantity in the sample of one or more of the cervical cancer-associated proteins described herein. As used herein, "means for detecting" in one embodiment includes one or more antibodies specific for these proteins and means for detecting the binding of the antibodies to these proteins by, e.g., a standard sandwich immunoassay as described herein.

Where the presence of a protein within a cell is to be detected, e.g., as from a tissue sample, the kit also may comprise means for disrupting the cell structure so as to expose intracellular proteins.

#### 2.C. Nucleic Acid-based Assays.

The presence of a cervical cancer in an individual also may be determined by detecting, in a tissue or body fluid sample, a nucleic acid molecule encoding a cervical cancer-associated protein. Using methods well known to those of ordinary skill in the art, the cervical cancer-associated proteins of the invention may be sequenced, and then, based on the determined sequence, oligonucleotide probes designed for screening a cDNA library (see, for example, Maniatis *et al.* (1989) *supra*).

A target nucleic acid molecule encoding a marker cervical cancer-associated protein may be detected using a labeled binding moiety, capable of specifically binding the target nucleic acid.

The binding moiety may comprise, for example, a protein, a nucleic acid or a peptide nucleic acid. Additionally, a target nucleic acid, such as an mRNA encoding a cervical cancer-associated protein, may be detected by conducting, for example, a Northern blot analysis using labeled oligonucleotides, e.g., nucleic acid fragments complementary to and capable of hybridizing specifically with at least a portion of a target nucleic acid. While any length oligonucleotide may be utilized to hybridize an mRNA transcript, oligonucleotides typically within the range of 8-100



nucleotides, preferably within the range of 15-50 nucleotides, are envisioned to be most useful in standard hybridization assays.

The oligonucleotide selected for hybridizing to the target nucleic acid, whether synthesized  
5 chemically or by recombinant DNA methodologies, is isolated and purified using standard techniques and then preferably labeled (e.g., with  $^{35}\text{S}$  or  $^{32}\text{P}$ ) using standard labeling protocols. A sample containing the target nucleic acid then is run on an electrophoresis gel, the dispersed nucleic acids transferred to a nitrocellulose filter and the labeled oligonucleotide exposed to the filter under suitable hybridizing conditions, e.g. 50% formamide, 5 X SSPE, 2 X Denhardt's  
10 solution, 0.1% SDS at 42°C, as described in Maniatis *et al.* (1989) *supra*. Other useful procedures known in the art include solution hybridization, and dot and slot RNA hybridization. The amount of the target nucleic acid present in a sample optionally then is quantitated by measuring the radioactivity of hybridized fragments, using standard procedures known in the art.

15 In addition, oligonucleotides also may be used to identify other sequences encoding members of the target protein families. The methodology also may be used to identify genetic sequences associated with the nucleic acid sequences encoding the proteins described herein, e.g., to identify non-coding sequences lying upstream or downstream of the protein coding sequence, and which may play a functional role in expression of these genes. Additionally,  
20 binding assays may be conducted to identify and detect proteins capable of a specific binding interaction with a nucleic acid encoding a cervical cancer-associated protein, which may be involved e.g., in gene regulation or gene expression of the protein. In a further embodiment, the assays described herein may be used to identify and detect nucleic acid molecules comprising a sequence capable of recognizing and being specifically bound by a cervical cancer-associated  
25 protein.

In addition, it is anticipated that using a combination of appropriate oligonucleotide primers, i.e., more than one primer, the skilled artisan may determine the level of expression of a target gene *in vivo* by standard polymerase chain reaction (PCR) procedures, for example, by

quantitative PCR. Conventional PCR based assays are discussed, for example, in Innes *et al* (1990) "*PCR Protocols; A guide to methods and Applications*", Academic Press and Innes *et al* (1995) "*PCR Strategies*" Academic Press, San Diego, CA.

5    **3. Identification of Proteins Which Interact In Vivo With Cervical Cancer-associated Proteins.**

10    In addition, it is contemplated that the skilled artisan, using procedures like those described hereinbelow, may identify other molecules which interact *in vivo* with the cervical cancer-associated proteins described herein. Such molecules also may provide possible targets for chemotherapy.

15    By way of example, cDNA encoding proteins or peptides capable of interacting with cervical cancer-associated proteins can be determined using a two-hybrid assay, as reported in Durfee *et al.* (1993) *Genes & Develop.* 7: 555-559, the disclosure of which is incorporated herein by reference. The principle of the two hybrid system is that noncovalent interaction of two proteins triggers a process (transcription) in which these proteins normally play no direct role, because of their covalent linkage to domains that function in this process. For example, in the two-hybrid assay, detectable expression of a reporter gene occurs when two fusion proteins, one comprising a DNA-binding domain and one comprising a transcription initiation domain,  
20    interact.

25    The skilled artisan can use a host cell that contains one or more reporter genes, such as yeast strain Y153, reported in Durfee *et al.* (1993) *supra*. This strain carries two chromosomally located reporter genes whose expression is regulated by Gal4. A first reporter gene, is the *E. coli lacZ* gene under the control of the *Gal4* promoter. A second reporter gene is the selectable *HIS3* gene. Other useful reporter genes may include, for example, the luciferase gene, the *LEU2* gene, and the *GFP* (Green Fluorescent Protein) gene.

30    Two sets of plasmids are used in the two hybrid system. One set of plasmids contain DNA encoding a *Gal4* DNA-binding domain fused in frame to DNA encoding a cervical cancer-

associated protein. The other set of plasmids contain DNA encoding a *Gal4* activation domain fused to portions of a human cDNA library constructed from human lymphocytes. Expression from the first set of plasmids result in a fusion protein comprising a Gal4 DNA-binding domain and a cervical cancer-associated protein. Expression from the second set of plasmids produce a transcription activation protein fused to an expression product from the lymphocyte cDNA library. When the two plasmids are transformed into a gal-deficient host cell, such as the yeast Y153 cells described above, interaction of the *Gal* DNA binding domain and transcription activation domain occurs only if the cervical cancer-associated protein fused to the DNA binding domain binds to a protein expressed from the lymphocyte cDNA library fused to the transcription activating domain. As a result of the protein-protein interaction between the cervical cancer-associated protein and its *in vivo* binding partner detectable levels of reporter gene expression occur.

In addition to identifying molecules which interact *in vivo* with the cervical cancer-associated proteins, the skilled artisan may also screen for molecules, for example, small molecules which alter or inhibit specific interaction between a cervical cancer-associated protein and its *in vivo* binding partner.

For example, host cell can be transfected with DNA encoding a suitable DNA binding domain/cervical cancer-associated protein hybrid and a translation activation domain/putative cervical cancer-associated protein binding partner, as disclosed above. The host cell also contains a suitable reporter gene in operative association with a *cis*-acting transcription activation element that is recognized by the transcription factor DNA binding domain. The level of reporter gene expressed in the system is assayed. Then, the host cell is exposed to a candidate molecule and the level of reporter gene expression is detected. A reduction in reporter gene expression is indicative of the candidate's ability to interfere with complex formation or stability with respect to the cervical cancer-associated protein and its *in vivo* binding partner. As a control, the candidate molecule's ability to interfere with other, unrelated protein-protein complexes is also tested. Molecules capable of specifically interfering with a cervical cancer-associated protein/binding partner interaction, but not other protein-protein interactions, are identified as

candidates for production and further analysis. Once a potential candidate has been identified, its efficacy in modulating cell cycling and cell replication can be assayed in a standard cell cycle model system.

5       Candidate molecules can be produced as described hereinbelow. For example, DNA encoding the candidate molecules can be inserted, using conventional techniques well described in the art (see, for example, Maniatis (1989) *supra*) into any of a variety of expression vectors and transfected into an appropriate host cell to produce recombinant proteins, including both full length and truncated forms. Useful host cells include *E. coli*, *Saccharomyces cerevisiae*, *Pichia*  
10 *pastoris*, the insect/baculovirus cell system, myeloma cells, and various other mammalian cells. The full length forms of such proteins are preferably expressed in mammalian cells, as disclosed herein. The nucleotide sequences also preferably include a sequence for targeting the translated sequence to the nucleus, using, for example, a sequence encoding the eight amino acid nucleus targeting sequence of the large T antigen, which is well characterized in the art. The vector can  
15 additionally include various sequences to promote correct expression of the recombinant protein, including transcription promoter and termination sequences, enhancer sequences, preferred ribosome binding site sequences, preferred mRNA leader sequences, preferred protein processing sequences, preferred signal sequences for protein secretion, and the like. The DNA sequence encoding the gene of interest can also be manipulated to remove potentially inhibiting sequences  
20 or to minimize unwanted secondary structure formation. As will be appreciated by the practitioner in the art, the recombinant protein can also be expressed as a fusion protein.

After translation, the protein can be purified from the cells themselves or recovered from the culture medium. The DNA can also include sequences which aid in expression and/or  
25 purification of the recombinant protein. The DNA can be expressed directly or can be expressed as part of a fusion protein having a readily cleavable fusion junction.

The DNA may also be expressed in a suitable mammalian host. Useful hosts include fibroblast 3T3 cells, (e.g., NIH 3T3, from CRL 1658) COS (simian kidney ATCC, CRL-1650) or  
30 CHO (Chinese hamster ovary) cells (e.g., CHO-DXB11, from Chasin (1980) *Proc. Nat'l. Acad.*

*Sci. USA* 77 :4216-4222), mink-lung epithelial cells (MVLu), human foreskin fibroblast cells, human glioblastoma cells, and teratocarcinoma cells. Other useful eukaryotic cell systems include yeast cells, the insect/baculovirus system or myeloma cells.

5 In order to express a candidate molecule, the DNA is subcloned into an insertion site of a suitable, commercially available vector along with suitable promoter/enhancer sequences and 3' termination sequences. Useful promoter/enhancer sequence combinations include the CMV promoter (human cytomegalovirus (MIE) promoter) present, for example, on pCDM8, as well as the mammary tumor virus promoter (MTV) boosted by the Rous sarcoma virus LTR enhancer  
10 sequence (e.g., from Clontech, Inc., Palo Alto). A useful inducible promoter includes, for example, A  $Zn^{2+}$  inducible promoter, such as the  $Zn^{2+}$  metallothionein promoter (Wrana et al. (1992) Cell 71: 1003-1014) Other inducible promoters are well known in the art and can be used with similar success. Expression also can be further enhanced using *trans*-activating enhancer sequences. The plasmid also preferably contains an amplifiable marker, such as DHFR  
15 under suitable promoter control, e.g., SV40 early promoter (ATCC #37148). Transfection, cell culturing, gene amplification and protein expression conditions are standard conditions, well known in the art, such as are described, for example in Ausubel *et al.*, ed.,(1989) "*Current Protocols in Molecular Biology*", John Wiley & Sons, NY. Briefly, transfected cells are cultured in medium containing 5-10% dialyzed fetal calf serum (dFCS), and stably transfected high  
20 expression cell lines obtained by amplification and subcloning and evaluated by standard Western and Northern blot analysis. Southern blots also can be used to assess the state of integrated sequences and the extent of their copy number amplification.

The expressed candidate protein is then purified using standard procedures. A currently  
25 preferred methodology uses an affinity column, such as a ligand affinity column or an antibody affinity column. The column is then washed, and the candidate molecules selectively eluted in a gradient of increasing ionic strength, changes in pH, or addition of mild detergent. It is appreciated that in addition to the candidate molecules which bind to the cervical cancer-associated proteins, the cervical cancer associated proteins themselves may likewise be produced  
30 using such recombinant DNA technologies.

#### 4. Cervical Cancer Therapy and Methods for Monitoring Therapy.

The skilled artisan, after identification of cervical cancer-associated proteins and proteins  
5 which interact with the cervical cancer-associated proteins, can develop a variety of therapies for  
treating cervical cancer. Because the marker proteins described herein are present at detectably  
higher levels in cervical cancer cells relative to normal cervical cells, the skilled artisan may  
employ, for example, the marker proteins and/or nucleic acids encoding the marker proteins as  
target molecules for a cancer chemotherapy.

##### 4.A. Anti-sense-based Therapeutics.

A particularly useful cancer therapeutic envisioned is an oligonucleotide or peptide  
nucleic acid sequence complementary and capable of hybridizing under physiological conditions  
15 to part, or all, of the gene encoding the marker protein or to part, or all, of the transcript encoding  
the marker protein thereby to reduce or inhibit transcription and/or translation of the marker  
protein gene. Alternatively, the same technologies may be applied to reduce or inhibit  
transcription and/or translation of the proteins which interact with the cervical cancer-associated  
proteins.

Anti-sense oligonucleotides have been used extensively to inhibit gene expression in  
normal and abnormal cells. See, for example, Stein *et al.* (1988) *Cancer Res.* 48: 2659-2668, for  
a pertinent review of anti-sense theory and established protocols. In addition, the synthesis and  
use of peptide nucleic acids as anti-sense-based therapeutics are described in PCT publications  
25 PCT/EP92/01219 published November 26, 1992, PCT/US92/10921 published June 24, 1993,  
and PCT/US94/013523 published June 1, 1995, the disclosures of which are incorporated herein  
by reference. Accordingly, the anti-sense-based therapeutics may be used as part of  
chemotherapy, either alone or in combination with other therapies.

Anti-sense oligonucleotide and peptide nucleic acid sequences are capable of hybridizing to a gene and/or mRNA transcript and, therefore, may be used to inhibit transcription and/or translation of the protein described herein. It is appreciated, however, that oligoribonucleotide sequences generally are more susceptible to enzymatic attack by ribonucleases than are deoxyribonucleotide sequences. Hence, oligodeoxyribonucleotides are preferred over oligoribonucleotides for *in vivo* therapeutic use. It is appreciated that the peptide nucleic acid sequences, unlike regular nucleic acid sequences, are not susceptible to nuclease degradation and, therefore, are likely to have greater longevity *in vivo*. Furthermore, it is appreciated that peptide nucleic acid sequences bind complementary single stranded DNA and RNA strands more strongly than corresponding DNA sequences (see, for example, PCT/EP92/20702 published November 26, 1992). Accordingly, peptide nucleic acid sequences are preferred for *in vivo* therapeutic use.

Therapeutically useful anti-sense oligonucleotides or peptide nucleic acid sequences may be synthesized by any of the known chemical oligonucleotide and peptide nucleic acid synthesis methodologies well known and thoroughly described in the art. Alternatively, a complementary sequence to part or all of the natural mRNA sequence may be generated using standard recombinant DNA technologies.

Since the complete nucleotide sequence encoding the entire marker protein as well as additional 5' and 3' untranslated sequences are known for each of the marker proteins and/or can be determined readily using techniques well known in the art, anti-sense oligonucleotides or peptide nucleic acids which hybridize with any portion of the mRNA transcript or non-coding sequences may be prepared using conventional oligonucleotide and peptide nucleic acid synthesis methodologies.

Oligonucleotides complementary to, and which hybridizable with any portion of the mRNA transcripts encoding the marker proteins are, in principle, effective for inhibiting translation of the target proteins as described herein. For example, as described in U.S. Pat. No. 5,098,890, issued March 24, 1992, the disclosure of which is incorporated herein by reference,

oligonucleotides complementary to mRNA at or near the translation initiation codon site may be used to inhibit translation. Moreover, it has been suggested that sequences that are too distant in the 3' direction from the translation initiation site may be less effective in hybridizing the mRNA transcripts because of potential ribosomal "read-through", a phenomenon whereby the ribosome is postulated to unravel the anti-sense/sense duplex to permit translation of the message.

A variety of sequence lengths of oligonucleotide or peptide nucleic acid may be used to hybridize to mRNA transcripts. However, very short sequences (e.g., sequences containing less than 8-15 nucleobases) may bind with less specificity. Moreover, for *in vivo* use, short oligonucleotide sequences may be particularly susceptible to enzymatic degradation. Peptide nucleic acids, as mentioned above, likely are resistant to nuclease degradation. Where oligonucleotide and peptide nucleic acid sequences are to be provided directly to the cells, very long sequences may be less effective at inhibition because of decreased uptake by the target cell. Accordingly, where the oligonucleotide or peptide nucleic acid is to be provided directly to target cells, oligonucleotide and/or peptide nucleic acid sequences containing about 8-50 nucleobases, and more preferably 15-30 nucleobases, are envisioned to be most advantageous.

An alternative means for providing anti-sense oligonucleotide sequences to a target cell is gene therapy where, for example, a DNA sequence, preferably as part of a vector and associated with a promoter, is expressed constitutively inside the target cell. Recently, Oeller *et al.* (Oeller *et al.* (1992) *Science* 254: 437-539, the disclosure of which is incorporated herein by reference) described the *in vivo* inhibition of the ACC synthase enzyme using a constitutively expressible DNA sequence encoding an anti-sense sequence to the full length ACC synthase transcript. Accordingly, where the anti-sense oligonucleotide sequences are provided to a target cell indirectly, for example, as part of an expressible gene sequence to be expressed within the cell, longer oligonucleotide sequences, including sequences complementary to substantially all the protein coding sequence, may be used to advantage.

Finally, therapeutically useful oligonucleotide sequences envisioned also include not only native oligomers composed of naturally occurring nucleotides, but also those comprising



modified nucleotides to, for example, improve stability and lipid solubility and thereby enhance cellular uptake. For example, it is known that enhanced lipid solubility and/or resistance to nuclease digestion results by substituting a methyl group or sulfur atom for a phosphate oxygen in the internucleotide phosphodiester linkage. Phosphorothioates ("S-oligonucleotides" wherein a phosphate oxygen is replaced by a sulfur atom), in particular, are stable to nuclease cleavage, are soluble in lipids, and are preferred, particularly for direct oligonucleotide administration. S-oligonucleotides may be synthesized chemically using conventional synthesis methodologies well known and thoroughly described in the art.

Preferred synthetic internucleoside linkages include phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidate, and carboxymethyl esters. Furthermore, one or more of the 5'-3' phosphate group may be covalently joined to a low molecular weight (e.g., 15-500 Da) organic group, including, for example, lower alkyl chains or aliphatic groups (e.g., methyl, ethyl, propyl, butyl), substituted alkyl and aliphatic groups (e.g., aminoethyl, aminopropyl, aminohydroxyethyl, aminohydroxypropyl), small saccharides or glycosyl groups. Other low molecular weight organic modifications include additions to the internucleoside phosphate linkages such as cholesteryl or diamine compounds with varying numbers of carbon residues between the amino groups and terminal ribose. Oligonucleotides with these linkages or with other modifications can be prepared using methods well known in the art (see, for example, U.S. Pat. No. 5,149,798).

Suitable oligonucleotide and or peptide nucleic acid sequences which inhibit transcription and/or translation of the marker proteins can be identified using standard *in vivo* assays well characterized in the art. Preferably, a range of doses is used to determine effective concentrations for inhibition as well as specificity of hybridization. For example, in the cases of an oligonucleotide, a dose range of 0-100µg oligonucleotide/ml may be assayed. Further, the oligonucleotides may be provided to the cells in a single transfection, or as part of a series of transfections. Anti-sense efficacy may be determined by assaying a change in cell proliferation over time following transfection, using standard cell counting methodology and/or by assaying for reduced expression of marker protein, e.g., by immunofluorescence. Alternatively, the ability

of cells to take up and use thymidine is another standard means of assaying for cell division and may be used here, e.g., using  $^3\text{H}$  thymidine. Effective anti-sense inhibition should inhibit cell division sufficiently to reduce thymidine uptake, inhibit cell proliferation, and/or reduce detectable levels of marker proteins.

5

It is anticipated that therapeutically effective oligonucleotide or peptide nucleic acid concentrations may vary according to the nature and extent of the neoplasm, the particular nucleobase sequence used, the relative sensitivity of the neoplasm to the oligonucleotide or peptide nucleic acid sequence, and other factors. Useful ranges for a given cell type and oligonucleotide and/or peptide nucleic acid may be determined by performing standard dose range experiments. Dose range experiments also may be performed to assess toxicity levels for normal and malignant cells. It is contemplated that useful concentrations may range from about 1 to 100  $\mu\text{g}/\text{ml}$  per  $10^5$  cells.

15 For *in vivo* use, the anti-sense oligonucleotide or peptide nucleic acid sequences may be combined with a pharmaceutical carrier, such as a suitable liquid vehicle or excipient, and optionally an auxiliary additive or additives. Liquid vehicles and excipients are conventional and are available commercially. Illustrative thereof are distilled water, physiological saline, aqueous solutions of dextrose, and the like. For *in vivo* cancer therapies, the anti-sense sequences preferably can be provided directly to malignant cells, for example, by injection directly into the tumor. Alternatively, the oligonucleotide or peptide nucleic acid may be administered systemically, provided that the anti-sense sequence is associated with means for directing the sequences to the target malignant cells.

25 In addition to administration with conventional carriers, the anti-sense oligonucleotide or peptide nucleic acid sequences may be administered by a variety of specialized oligonucleotide delivery techniques. For example, oligonucleotides may be encapsulated in liposomes, as described in Mannino *et al.* (1988) *BioTechnology* 6: 682, and Felgner *et al.* (1989) *Bethesda Res. Lab. Focus* 11:21. Lipids useful in producing liposomal formulations include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile

acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art (see, for example, in U.S. Pat. No. 4,235,871; U.S. Pat. No. 4,501,728; U.S. Pat. No. 4,837,028; and U.S. Pat. No. 4,737,323). The pharmaceutical composition of the invention may further include compounds such as cyclodextrins and the like which enhance delivery of oligonucleotides into cells. When the composition is not administered systemically but, rather, is injected at the site of the target cells, cationic detergents (e.g. Lipofectin) may be added to enhance uptake. In addition, reconstituted virus envelopes have been successfully used to deliver RNA and DNA to cells (see, for example, Arad *et al.* (1986) *Biochem. Biophys. Acta.* 859: 88-94).

For therapeutic use *in vivo*, the anti-sense oligonucleotide and/or peptide nucleic acid sequences are administered to the individual in a therapeutically effective amount, for example, an amount sufficient to reduce or inhibit target protein expression in malignant cells. The actual dosage administered may take into account whether the nature of the treatment is prophylactic or therapeutic in nature, the age, weight, health of the patient, the route of administration, the size and nature of the malignancy, as well as other factors. The daily dosage may range from about 0.01 to 1,000 mg per day. Greater or lesser amounts of oligonucleotide or peptide nucleic acid sequences may be administered, as required. As will be appreciated by those skilled in the medical art, particularly the chemotherapeutic art, appropriate dose ranges for *in vivo* administration would be routine experimentation for a clinician. As a preliminary guideline, effective concentrations for *in vitro* inhibition of the target molecule may be determined first.

#### **4.B. Binding Protein-based Therapeutics.**

As mentioned above, a cancer marker protein or a protein that interacts with the cancer marker protein may be used as a target for chemotherapy. For example, a binding protein designed to bind the marker protein essentially irreversibly can be provided to the malignant cells, for example, by association with a ligand specific for the cell and known to be absorbed by the cell. Means for targeting molecules to particular cells and cell types are well described in the chemotherapeutic art.

Binding proteins may be obtained and tested using technologies well known in the art. For example, the binding portions of antibodies may be used to advantage. It is contemplated, however, that intact antibodies or BABS, which preferably, have been humanized may be used in the practice of the invention. As used herein, the term "humanized" is understood to mean a process whereby the framework region sequences of a non-human immunoglobulin variable region are replaced by human variable region sequences. Accordingly, it is contemplated that such humanized binding proteins will elicit a weaker immune response than their unhumanized counterparts. Particularly useful are binding proteins identified with high affinity for the target protein, e.g., greater than about  $10^9 \text{ M}^{-1}$ . Alternatively, DNA encoding the binding protein may be provided to the target cell as part of an expressible gene to be expressed within the cell following the procedures used for gene therapy protocols well described in the art. See, for example, U.S. Patent No. 4,497,796, and "Gene Transfer", Vijay R. Baichwal, ed., (1986). It is anticipated that, once bound by binding protein, the target protein will be inactivated or its biological activity reduced thereby inhibiting or retarding cell division.

As described above, suitable binding proteins for *in vivo* use, may be combined with a suitable pharmaceutical carrier, such as physiological saline or other useful carriers well characterized in the medical art. The pharmaceutical compositions may be provided directly to malignant cells, for example, by direct injection, or may be provided systemically, provided the binding protein is associated with means for targeting the protein to target cells. Finally, suitable dose ranges and cell toxicity levels may be assessed using standard dose range experiments.

Therapeutically effective concentrations may range from about 0.01 to about 1,000 mg per day. As described above, actual dosages administered may vary depending, for example, on the nature of the malignancy, the age, weight and health of the individual, as well as other factors.

#### 4.C. Small Molecule-based Therapeutics.

After having isolating cervical cancer-associated nuclear matrix proteins, the skilled artisan can, using methodologies well known in the art, can screen small molecule libraries

(either peptide or non-peptide based libraries) to identify candidate molecules that reduce or inhibit the biological function of the cervical cancer-associated proteins. The small molecules preferably accomplish this function by reducing the *in vivo* expression of the target molecule, or by interacting with the target molecule thereby to inhibit either the biological activity of the target molecule or an interaction between the target molecule and its *in vivo* binding partner.

It is contemplated that, once the candidate small molecules have been elucidated, skilled artisan may enhance the efficacy of the small molecule using rational drug design methodologies well known in the art. Alternatively, the skilled artisan may use a variety of computer programs which assist the skilled artisan to develop quantitative structure activity relationships (QSAR) which further to assist the design of additional candidate molecules *de novo*. Once identified, the small molecules may be produced in commercial quantities and subjected to the appropriate safety and efficacy studies.

It is contemplated that the screening assays may be automated thereby facilitating the screening of a large number of small molecules at the same time. Such automation procedures are within the level of skill in the art of drug screening and, therefore, are not discussed herein. Candidate peptide based small molecules may be produced by expression of an appropriate nucleic acid sequence in a host cell or using synthetic organic chemistries. Similarly, non-peptidyl-based small molecules may be produced using conventional synthetic organic chemistries well known in the art.

As described above, for *in vivo* use, the identified small molecules may be combined with a suitable pharmaceutical carrier, such as physiological saline or other useful carriers well characterized in the medical art. The pharmaceutical compositions may be provided directly to malignant cells, for example, by direct injection, or may be provided systemically, provided the binding protein is associated with means for targeting the protein to target cells. Finally, suitable dose ranges and cell toxicity levels may be assessed using standard dose range experiments. As described above, actual dosages administered may vary depending, for example, on the nature of the malignancy, the age, weight and health of the individual, as well as other factors.

#### 4.D. Methods for Monitoring the Status of Cervical Cancer in an Individual.

5 The progression of the cervical cancer or the therapeutic efficacy of chemotherapy may  
be measured using procedures well known in the art. For example, the efficacy of a particular  
chemotherapeutic agent can be determined by measuring the amount of a cervical cancer-  
associated protein released from cervical cancer cells undergoing cell death. As reported in PCT  
publication PCT/US92/09220, published May 13, 1993, incorporated by reference herein,  
soluble nuclear matrix proteins and fragments thereof are released by cells upon cell death. Such  
10 soluble nuclear matrix proteins can be quantitated in a body fluid and used to monitor the degree  
or rate of cell death in a tissue.

For example, the concentration of a body fluid-soluble nuclear matrix proteins or a  
fragment thereof released from cells is compared to standards from healthy, untreated tissue.  
15 Fluid samples are collected at discrete intervals during treatment and compared to the standard.  
It is contemplated that changes in the level of a body fluid soluble cervical cancer-associated  
protein, will be indicative of the efficacy of treatment (that is, the rate of cancer cell death). It is  
contemplated that the release of body fluid soluble interior nuclear matrix proteins can be  
measured in blood, plasma, urine, sputum, vaginal secretion, and breast exudate.

20 Where the assay is used to monitor tissue viability or progression of cervical cancer, the  
step of detecting the presence and abundance of the marker protein or its transcript in samples of  
interest is repeated at intervals and these values then are compared, the changes in the detected  
concentrations reflecting changes in the status of the tissue. For example, an increase in the level  
25 of cervical cancer-associated proteins may correlate with progression of the cervical cancer.  
Where the assay is used to evaluate the efficacy of a therapy, the monitoring steps occur  
following administration of the therapeutic agent or procedure (e.g., following administration of  
a chemotherapeutic agent or following radiation treatment). Similarly, a decrease in the level of  
cervical cancer-associated proteins may correlate a regression of the cervical cancer.

Thus, cervical cancer may be identified by the presence of cervical cancer-associated proteins as taught herein. Once identified, the cervical cancer may be treated using compounds which reduce *in vivo* the expression and/or biological activity of the cervical cancer-associated proteins. Furthermore, the methods provided herein can be used to monitor the progression of the disease and/or treatment of the disease. The following non limiting examples provide details of the isolation and characterization of cervical cancer-associated proteins and methods for their use in the detection of cervical cancer.

### *Example 1*

#### **Isolation of Cervical Cancer-Associated Nuclear Matrix Proteins From Cervical Cancer Tissue Samples and Cell Lines.**

Cervical cancer-associated proteins were identified by comparing silver stained 2-D gel patterns of proteins isolated from normal and cancerous cervical cells.

Fresh cervical carcinoma tissue was obtained from patients undergoing hysterectomy for clinically localized (stage IB, II or III, International Federation of Gynecology and Obstetrics or FIGO classification) carcinomas of the cervix from the Instituto Nacional de Cancerologia in Mexico City, Mexico, in accordance with Scientific and Ethics Committee Review Board approval. A small number of tumor tissues were obtained under Institutional Review Board approval from the Pittsburgh Cancer Center (Pittsburgh, PA). Normal cervical tissue was obtained under Institutional Review Board approval from patients undergoing hysterectomy for causes unrelated to abnormal cervical histopathology, via the Cooperative Human Tissue Network (Columbus, OH). Clinical staging and tumor histopathology for twenty patients who provided tissue samples for use in these experiments are shown in Table 1. With the exception of one case of adenosquamous carcinoma, all of the tumors were squamous cell carcinomas. A majority of these were of the large cell non-keratinizing type. All the patients had localized disease with clinical stages ranging from IB to IIIB (Table 1).

**TABLE 1. Patient Age, Clinical Staging and Histopathology.**

Case Number	Patient Age	FIGO Stage	Histopathological Diagnosis
1	37	IB	LCNKS <sup>†</sup>
2	49	IB	LCKS <sup>‡</sup>
3	32	IB	Squamous, mod. well diff.*
4	60	IIA	LCNKS
5	63	III	Adenosquamous
6	35	IB	LCKS
7	44	IIIB	LCNKS
8	31	IB	Squamous, poorly diff. <sup>§</sup>
9	31	IB	LCNKS
10	38	IIB	LCKS
11	65	IIB	LCNKS
12	35	IB	LCNKS
13	43	IB	LCNKS
14	65	III	LCNKS
15	52	IIB	LCKS
16	47	III	LCNKS
17	33	IB	LCNKS
18	51	IIIB	LCNKS
19	45	IIB	LCNKS
20	39	IIB	LCNKS

5

FIGO Stage

FIGO Stage	IB	IIA	IIB	III	IIIB
<i>n</i>	9	1	5	3	2

10

<sup>†</sup>, Large cell nonkeratinizing squamous cell carcinoma

<sup>‡</sup>, Large cell keratinizing squamous cell carcinoma

<sup>\*</sup>, Squamous cell carcinoma, moderately well differentiated

<sup>§</sup>, Squamous cell carcinoma, poorly differentiated

15

Fresh tissue was obtained during surgery, placed into transport medium (RPMI 1640 supplemented with gentamicin and 10% fetal calf serum (GIBCO)), packed in ice, and shipped to Matritech, Inc. by overnight carrier. In a small number of cases where immediate shipment could not be arranged, tissues specimens were snap-frozen in liquid nitrogen and sent on dry ice



to Matritech, Inc. by overnight carrier. Minimum size of tissue specimens was 0.2 gram. Diagnosis was obtained from pathology reports that accompanied each specimen.

Nuclear matrix proteins were isolated from cervical cancer tissue using a modification of the method of Fey *et al.* (1986) *supra*. Fresh cervical cancer tissue specimens, ranging in size from about 0.2 g to about 1.0 g, were obtained from 20 different patients. Tissue specimens were minced into small (1 mm<sup>3</sup>) pieces and homogenized with a Teflon pestle on ice and treated with a buffered solution containing 0.5% Triton-X-100, vanadyl riboside complex (RNAase inhibitor, Five Prime-Three Prime, Inc.) plus a protease inhibitor cocktail containing phenylmethyl sulfonyl fluoride (Sigma Chemical Co.), aprotinin and leupeptin (Boehringer Mannheim), to remove lipids and soluble proteins.

Stromal aggregates were removed by filtering the homogenate through 250 micron Nitex nylon screen (Tetko, Inc.) followed by a centrifugation step (600 x g, 4°C, 5min). Soluble cytoskeletal proteins were removed by incubating the pellet in an extraction buffer containing 250 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5% Triton X-100, vanadyl riboside complex and protease inhibitor cocktail on ice for 10 minutes followed by centrifugation (600 x g, 4°C, 5min).

Chromatin was removed by incubating the pellet in DNAase (100 mg/mL, Boehringer-Mannheim) in a buffered solution containing protease inhibitor cocktail for 45 minutes at 25°C. The remaining pellet fraction, which contained nuclear matrix proteins and intermediate filaments, was solubilized in disassembly buffer containing 8 M urea, protease inhibitor cocktail and 1% (vol/vol) 2-mercaptoethanol. Insoluble contaminants, primarily carbohydrates and extracellular matrix were removed by ultracentrifugation (163,000 x g, 20°C, 1 hr). Intermediate filaments were allowed to reassemble upon removal of urea by dialysis in an assembly buffer containing 150 mM KCl, 24 mM imidazole HCl, 5 mM MgCl<sub>2</sub>, 0.125 mM EGTA and 2 mM dithiothreitol (DTT) with protease inhibitors and were removed by ultracentrifugation (109,000 x g, 15°C, 1.5 hr), leaving the nuclear matrix proteins in the supernatant fraction.

In addition, cervical cancer-associated proteins were isolated from CaSki, ME-180, C33A, HeLa (S3 subline), SiHa, C4-1, C4-11, and HT-3 cervical tumor cell lines. Each cell line was obtained from the American Type Culture Collection (ATCC) and maintained at 37°C in 5% CO<sub>2</sub> in Dulbecco's Modified Eagles Medium supplemented with 10% fetal calf serum, gentamicin, fungizone and 0.12% SeraExtend (Irvine Scientific). For nuclear matrix extraction studies, cells were grown to approximately 80% confluence in 10 stage cell culture factories (Nunc), harvested by trypsinization, counted and extracted in the same manner as homogenized tumor tissue. Protein concentration of nuclear matrix proteins was determined by the Coomassie Plus Protein Assay Kit (Pierce Chemical) using a bovine gamma globulin standard. Proteins were immediately precipitated in 80% ethanol and stored at -80°C until use.

The resulting nuclear matrix proteins were next characterized by high-resolution two-dimensional gel electrophoresis according to the procedure of O'Farrell (1975) *J. Biol. Chem.* 250: 4007-4021 (1975), on an Investigator 2-D system (Oxford Glycosystems, Bedford, MA). Nuclear matrix proteins were solubilized for isoelectric focusing (IEF) analysis in sample buffer containing 9 M urea, 65 mM 3-[(cholamidopropyl)dimethylamino]-1-propanesulfate (CHAPS), 2.2% ampholytes, and 140 mM dithiothreitol (DTT). Two hundred micrograms of nuclear matrix proteins were loaded per gel.

One-dimensional isoelectric focusing was carried out for 18,000 volt-hours using 1 mm x 18 mm gel tubes. Following first dimension electrophoresis, gels were extruded from gel tubes, equilibrated for 2 minutes in a buffer containing 0.3 M Tris base, 0.075 M Tris-HCl, 3.0% SDS, 50 mM DTT, and 0.01% bromophenol blue and placed on top of 1 mm 10% Tris-glycine-SDS Duracryl (Oxford Glycosystems) high tensile strength polyacrylamide electrophoresis slab gels. Second dimension slab gels were electrophoresed at 16 Watts per gel and 12 °C constant temperature for approximately 5 hours. Molecular weight standards consisted of bovine albumin (M<sub>r</sub> 66,000), ovalbumin (M<sub>r</sub> 45,000), glyceraldehyde-3-phosphate dehydrogenase (M<sub>r</sub> 36,000), carbonic anhydrase (M<sub>r</sub> 29,000), bovine pancreatic trypsinogen (M<sub>r</sub> 24,000), and soybean trypsin inhibitor (M<sub>r</sub> 20,100) (Sigma Chemical Co.). Isoelectric points were determined using internal

control proteins with well-characterized isoelectric points. Following electrophoresis, gels were fixed in a solution containing 40% ethanol/10% acetic acid followed by treatment with a solution containing 0.5% glutaraldehyde. Gels were washed extensively and silver stained according to the method of Rabillioud *et al.* (Rabillioud *et al.* (1992) *Electrophoresis* 13: 429-439) and dried  
5 between sheets of cellophane paper.

Silver-stained gels were imaged using a MasterScan Biological Imaging System (CSP, Inc., Billerica, MA) according to the manufacturer's instructions. Digital filtering algorithms were used to remove both uniform and non-uniform background without removing critical image  
10 data. Two-D scan (TM) two-dimensional gel analysis and database software (version 3.1) using multiple Gaussian least-squares fitting algorithms were used to compute spot patterns into optimal-fit models of the data as reported by Olson *et al.* (1980) *Anal. Biochem.* 169: 49-70. Triangulation from the internal standards was used to precisely determine the molecular weight and isoelectric point of each target protein of interest. Interpretive densitometry was performed  
15 using specific software application modules to integrate the data into numeric and graphical reports for each gel being analyzed.

## Example 2

### Identification of Cervical Cancer-associated Nuclear Matrix Proteins Having Differential Appearance on 2-D Gels.

  
20

As described in the previous Example, 2-D gel electrophoresis patterns were obtained by fractionating proteins isolated from either normal or cancerous cervical cells. Figure 1a shows a typical cervical cancer-associated nuclear matrix protein pattern obtained from cervical cancer  
25 tissue. Figure 1b shows a typical gel pattern produced by nuclear matrix proteins obtained from a normal cervical tissue sample. Approximately 600 proteins were resolved per gel. Most of the proteins observed were always present, irrespective of the type of cervical tissue under investigation.

Comparison of Figures 1 and 2 reveals that, while most proteins in the cancer and non-cancer samples are identical, there are five proteins that are unique to the cervical cancer sample (labeled in Figure 1). The proteins, designated CvC-1 through CvC-5, were detected in 20 tissue samples obtained from patients diagnosed with cervical carcinoma but were not detected in cervical tissue isolated from a group of 10 normal individuals. Table 2 identifies proteins, designated CvC-1 through CvC-5, by their approximate molecular weight and isoelectric point. Both the molecular weight and isoelectric point values listed in Table 1 are approximate and accurate to within 2,000 Daltons for molecular weight and to within 0.2 pI units for isoelectric point. A detailed analysis to identify proteins common to normal cervical tissue but absent from cervical cancer tissue did not reveal any proteins that were specifically associated with normal cervical tissue.

***Table 2: Cervical Cancer-associated Proteins***

Peptide	Molecular Weight	Isoelectric Point	Cervical Cancer	Normal Cervical
CvC-1	69,408	5.78	+	-
CvC-2	53,752	5.54	+	-
CvC-3	47,887	5.60	+	-
CvC-4	46,006	5.07	+	-
CvC-5	44,864	6.61	+	-

In addition, the expression of nuclear matrix proteins isolated from cervical cancer cell lines was investigated, the results of which are summarized in Table 3, below. It is known that tumors of epithelial cell origin are characterized by the presence of stroma and other elements, such as those resulting from infiltrating inflammatory cells. Detection of nuclear matrix or matrix-associated proteins in tumor cell lines derived from cervical epithelial cell tumors reduces the possibility that the proteins are the result of stromal or other types of contamination of the nuclear matrix preparation.

2-D gel electrophoresis patterns were obtained from samples containing cervical cancer cells derived from cervical cancer cell lines. Figure 2a shows a cervical cancer-associated nuclear matrix protein pattern obtained from the cervical cancer cell line C33A. In Figure 2a, tumor-associated proteins CvC-2 and CvC-5 are encircled and identified with numbers 2 and 5. Figure 2b shows a gel pattern produced by nuclear matrix proteins obtained from the cervical cancer cell line CaSki a normal cervical tissue sample. In Figure 2b, tumor associated proteins CvC-1 and CvC-3 are encircled and identified with numbers 1 and 3.

Four of the five tumor-associated proteins (CvC1 to CvC-3 and CvC-5) were reproducibly detected in one or more cervical tumor cell lines (Figure 2, Table 3), confirming the epithelial origin of the proteins. Expression of the fifth protein, CvC-4, was variable but could be detected in the C33A tumor cell line (Table 3).

**Table 3. Cervical Carcinoma-associated Protein Expression in Cervical Tumor Cell Lines.**

Tumor cell line	Histopathology of tumor or origin	Nuclear matrix proteins expressed*				
		CvC-1	CvC-2	CvC-3	CvC-4	CvC-5
CaSKI <sup>†</sup>	Epidermoid	+	tr <sup>‡</sup>	+	-	+
SiHa	Squamous cell	-	tr	+++	-	+
HeLa	Adenocarcinoma	tr	-	+++	-	+
ME-180 <sup>†</sup>	Epidermoid	-	tr	+	-	+
C33A	Squamous cell	+	++	-	var <sup>§</sup>	+
C4-I	Squamous cell	tr	-	+++	-	+
C4-II	Squamous cell	-	-	-	-	tr
HT-3 <sup>†</sup>	Epidermoid	tr	-	+	-	tr

\* Nuclear matrix proteins were extracted from tumor cell lines obtained from the American Type Culture Collection using Fey and Penman extraction methodology.

<sup>†</sup> Tumor cell lines arising from metastatic epidermoid carcinoma originating from cervix.

<sup>‡</sup> Indicates low level expression, detected by silver stain.

<sup>§</sup> Indicates variable expression, detected by silver stain.

Two of the cervical cancer-associated proteins specific to cervical cancer cells were isolated and processed for microsequence analysis.

### Example 3

#### Characterization of Cervical Cancer-Associated Nuclear Matrix Protein Markers.

Two protein staining spots detectable on a 2-D gel corresponding to CvC-3 and CvC-5 were isolated, the protein harvested and subjected to microsequence analysis, as described hereinbelow.

For sequencing of the cervical cancer-associated polypeptides CvC-3 and CvC-5, the nuclear matrix fraction from HeLa cells were electrophoresed on two-dimensional gels as described above. Each gel was loaded with 300 micrograms of protein isolated by the nuclear matrix protein isolation procedure, as described above. Following the second-dimension of electrophoresis, proteins were visualized by reverse staining. Briefly, gels were soaked in 200 mM imidazole for 10 minutes, rinsed for 1 minute in water, followed by 1-2 minutes in 300 mM zinc chloride (Fernandez-Patron *et al.* (1992) *BioTechniques* 12: 564-573). After the protein-containing spots began to appear, the gels were placed in water, and the relevant gels spots excised. The isolated gel spots representing individual cervical cancer-associated polypeptides were pooled and destained by a 5 minute wash in 2% citric acid, followed by several washes in 100 mM Tris hydrochloride at pH 7.0 to raise the pH within the gel pieces.

Each set of pooled gel fragments was then diluted with an equal volume of 2X sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (250mM Tris-Cl, 2% SDS, 20% glycerol, 0.01% bromophenol blue and 10%  $\beta$ -mercaptoethanol, pH 6.8) and incubated at 75°C for 3 minutes. The gel fragment-containing samples were then cooled on ice and loaded onto a 4% polyacrylamide stacking/11% polyacrylamide separating SDS-PAGE gel, and electrophoresed in 1X Tank Buffer (24mM Tris-HCl, 192 mM glycine, 1% SDS, pH 8.3) to focus the gel spots into bands. Molecular weight markers (BioRad #161-0304) were used on

each gel to relate the observed molecular weights on the one-and two-dimensional gels.

Following electrophoresis, these gels were electroblotted onto Immobilon PVDF membranes (Oxford Glycosystems, Inc.) (Towbin *et al.* (1979) *Proc. Nat'l. Acad. Sci. USA* 76: 4350-4354) as modified by Matsudaira (Matsudaira *et al.* (1987) *J. Biol. Chem.* 262: 10035) for the mini-gel format. The membranes were then stained for 1 minute with Buffalo Black (0.1% in 1% acetic acid, 40% methanol) and rinsed with water. Regions of membrane containing polypeptide bands were excised with a clean scalpel.

The PVDF-bound polypeptides were then subjected to tryptic peptide mapping and microsequencing (Fernandez *et al.* (1994) *Analytical Biochem.* 218: 112-117) at the Microchemistry Facility at the Worcester Foundation for Biomedical Research using a Hewlett Packard Model 1090M HPLC. Sequence determinations were made on an Applied Biosystems ProCise Sequenator, and most were confirmed by MALDI-TOF mass spectrometry of individual peptides. Other peptides were identified by mass analysis alone, or mass analysis of carboxypeptidase-digested material.

#### Microsequence Analysis of CvC-3 Peptides.

Using the methodology described above, CvC-3 was isolated from approximately 120 two-dimensional gels of HeLa nuclear matrix and refocused on Immobilon-PVDF membrane for microsequence analysis. Although only one protein was observed by silver staining the 2-D gel location identified as CvC-3, refocusing of the protein on a one dimensional 11% minigel resulted in the resolution of two clearly separable protein bands. These proteins were labeled as CvC-3H and CvC-3L and submitted separately for microsequence analysis. Analysis of the tryptic maps indicates that two different proteins were contained in the two bands seen on the refocusing minigel, since little overlap was observed in the peak retention times of the two peptides.

Ten masses were detected by mass spectrometry from seven of the CvC-3H peaks.

Amino acid sequence was obtained for three peptides, two by Edman degradation and one by

carboxypeptidase-MALDI-TOF analysis. The sequences obtained for these peptides, shown in Table 4 match a protein known as IEF SSP 9502 or "novel human nuclear phosphoprotein". (Honore *et al.* (1994) *supra*; GenBank Accession #LO7758). The complete amino acid sequence for this protein, as derived from a gene sequence, is shown in SEQ. ID No.: 10. Seven other masses from peak fractions separated on the CvC-3H tryptic map also matched those of predicted tryptic fragments from this protein. Mass correlation data of tryptic peptides from CvC-3H are summarized in Table 4. The predicted molecular weight of the nuclear phosphoprotein, based upon its nucleotide sequence is 55 kDa, whereas its observed molecular weight by 2-D gel analysis is 79 kDa (Honore *et al.* (1994) *supra*).

**Table 4. Mass Correlation of CvC-3H-derived Tryptic Peptides**

Peak	Observed Mass (Da)	Predicted Mass (Da)	Delta	Sequence	SEQ. ID. No.	Protein
4	1110.64	1109.25	0.13%	PAASLAVHTDK	1	IEF SSP 9502
5	834.62	835.92	0.16%	FSGQIER	2	IEF SSP 9502
7	1056.57	1057.26	0.07%	RLIAEAKEK	3	IEF SSP 9502
8	1187.45	1185.37	0.18%	PSLVHSRDM	4	IEF SSP 9502
10	1774.73	1766.93	0.44%	<u>VWDISTVSSVNEAFGR*</u>	5	IEF SSP 9502
10	1802.22	1805.02	0.16%	LVLGSARNSSISGPFGR	6	IEF SSP 9502
11	2746.27	2743.02	0.12%	SDKPIFTLNAHNDEISGLDLSSQIK**	7	IEF SSP 9502
12	2412.23	2409.68	0.11%	<u>VQTLQEHFPFEAQTLSGSYDK*</u>	8	IEF SSP 9502
12	2475.13	2483.98	0.36%	MGVLCSSCCPDLPFIYAFGGQK	9	IEF SSP 9502

\* Underlining reflects sequences confirmed by Edman degradation.

\*\* Bolded underlining reflects sequence confirmed by carboxypeptidase digestion.

In addition, seven masses were detected by mass spectrometry from four peaks derived from tryptic digestion of CvC-3L. One of these was directly sequenced and was found to be identical to cytokeratin 17 (Trojanovsky *et al.* (1992), *supra*; GenBank Accession # Q04695). Six other masses from fractions separated on the CvC-3L tryptic map also matched those of predicted tryptic fragments of human cytokeratin 17. The amino acid sequence for this protein,



from Troyanovsky *et al.* (1992), *supra*, is shown in SEQ. ID No.: 18. Mass correlation data of tryptic peptides from CvC-3L are summarized in Table 5. The apparent molecular weight of CvC-3L (47.9 kDa) is consistent with the detection of a full length molecule of cytokeratin 17 (predicted molecular weight, 48 kDa) in cervical tumors.

5

**Table 5. Mass Correlation of CvC-3L-derived Tryptic Peptides.**

<u>Peak</u>	<u>Observed Mass (Da)</u>	<u>Predicted Mass (Da)</u>	<u>Delta</u>	<u>Sequence</u>	<u>SEQ. ID No.</u>	<u>Protein</u>
4	995.46	994.03	0.14%	DYSQYYR	11	Cytokeratin 17
4	1244.97	1242.34	0.21%	NHEEEMNALR	12	Cytokeratin 17
9	1518.03	1516.67	0.09%	<u>LLEGEDAHLTQYK*</u>	13	Cytokeratin 17
10	791.19	790.94	0.03%	ILNEMR	14	Cytokeratin 17
10	835.16	832.91	0.27%	SEISELR	15	Cytokeratin 17
12	1144.21	1144.21	0.00%	DAEDWFFSK	16	Cytokeratin 17
12	1187.57	1186.33	0.10%	LSVEADINGLR	17	Cytokeratin 17

\* Underlining reflects sequences confirmed by Edman degradation.

**Microsequence Analysis of CvC-5 Peptides.**

The gel spot identified as CvC-5 was collected from HeLa nuclear matrix from the same preparative two-dimensional gels that were used for the collection of CvC-3. Approximately 100 gel spots were collected as described and refocused on Immobilon-PVDF membrane for microsequence analysis. During the initial identification of tumor associated proteins it was noted that in some cervical tumors, two proteins appeared to migrate very close together in the location identified as CvC-5. Only one protein was clearly apparent. However, when the expression of this protein was examined in cervical tumor cell lines, 3 of 8 cell lines showed the presence of at least two proteins in the area defined by CvC-5 (Table 3). Without wishing to be bound by theory, one explanation for the apparent detection of only one protein in the CvC-5 gel

spot in many tumors is that one of the proteins may be more abundant, thereby masking the presence of other closely migrating proteins. When CvC-5 gel spots were pooled and refocused onto a one dimensional minigel, only one diffusely stained protein band was detected.

5 The tryptic map of the diffuse band containing the polypeptide components of the CvC-5 gel spot contained approximately 30 resolved peaks. Mass analysis was performed on 12 of these peaks and 30 masses were obtained. Six amino acid sequences were obtained by automated Edman degradation, revealing the presence of three distinct polypeptides. The first of these is a protein known as TDP-43 or TAR DNA binding protein (Out *et. al.* (1995) *supra*;  
10 GenBank Accession # U23731). The complete amino acid sequence, as derived from the gene sequence for this protein, is shown in SEQ. ID. No. 26. The apparent molecular weight of 43 kDa suggests identification of the intact protein in cervical tumors. Six other masses from fractions separated on the CvC-5 tryptic map also matched those of predicted tryptic fragments from this protein. Mass correlation data and peptide sequence data of tryptic peptides matching  
15 TDP-43 are shown in Table 6.

**Table 6. Mass Correlation of CvC-5 Derived Tryptic Peptides.**

<u>Peak</u>	<u>Observed Mass (Da)</u>	<u>Predicted Mass (Da)</u>	<u>Delta</u>	<u>Sequence</u>	<u>SEQ. ID. No.</u>	<u>Protein</u>
12	1729.01	1726.79	0.13%	FGGNPGGFGNQGGFGNSR	19	TDP43
13	655.72	653.78	0.30%	WCDCK	20	TDP43
13	834.24	833.89	0.04%	TTEQDLK	21	TDP43
14	682.63	681.79	0.12%	GFGFVR	22	TDP43
16	1511.88	1511.66	0.01%	LPNSKQSQDEPLR	23	TDP43
21	1280.01	1281.41	0.11%	KMDETDAASSAVK	24	TDP43
25	1342.84	1341.61	0.09%	<u>TSDLIVLGLPWK*</u>	25	TDP43

20 \* Underlining reflects sequences confirmed by Edman degradation.

Sequence information obtained for three peptides matched a nuclear pore protein known as nucleoporin or Nup358 (Wu *et al* (1995) *supra*, Gen Bank Accession # L41840). The complete amino acid sequence, as derived from the gene sequence, is shown in SEQ. ID. No.:34. Mass correlation data for five additional masses identified from the CvC-5 tryptic map which matched predicted tryptic fragments of Nup358 are shown in Table 7. The location of the sequences matching Nup358 suggests our isolation of a C-terminal fragment of the intact protein (M<sub>r</sub> 358 kDa) from cervical tumors.

**Table 7. Mass Correlation of CvC-5 Derived Tryptic Peptides.**

Peak	Observed Mass (Da)	Predicted Mass (Da)	Delta	Sequence	SEQ. ID No.	Protein
9	613.14	614.66	0.25%	NY <del>Y</del> R*	27	nup358
10	613.20	614.66	0.24%	NY <del>Y</del> R*	28	nup358
11	702.22	701.78	0.06%	VQEAQK	29	nup358
16	938.37	939.10	0.08%	EVADCFK	30	nup358
17	2459.64	2458.54	0.04%	<u>HDGTGGQSIYGD</u> KFEDENFDVK**	31	nup358
21	1419.00	1419.71	0.05%	<u>ITMELEXNIVPR</u> **	32	nup358
21	2773.58	2771.11	0.09%	<u>HTGPGLLSMANOQ</u> QNTNNXXFVIXLK**	33	nup358

\* Denotes a peptide that appeared in two adjacent HPLC fractions  
 \*\* Underlining reflects sequences confirmed by Edman degradation

The third polypeptide identified in the CvC-5 gel spot is a fragment of lamin A (Fisher *et al.* (1986), *supra*; GenBank Accession #M13452). Two sequences matching lamin A were obtained by Edman degradation (Table 8). Nine additional masses from fragments of the CvC-5 tryptic map match predicted masses of tryptic fragments from lamin A. Mass correlation data for these additional masses were shown in Table 8. The amino acid sequence for this protein, (Fisher *et al.* (1986) *supra*), is shown in SEQ. ID No.: 46.

**Table 8. Mass Correlation of CvC-5 derived Tryptic Peptides.**

<u>Peak</u>	<u>Observed Mass (Da)</u>	<u>Predicted Mass (Da)</u>	<u>Delta</u>	<u>Sequence</u>	<u>Seq. ID No.</u>	<u>Protein</u>
7	667.10	666.69	0.06%	EFESR	35	lamin A
8	569.50	568.63	0.15%	TYSAK*	36	lamin A
8	585.78	587.63	0.31%	LDNAR	37	lamin A
11	569.10	568.63	0.08%	TYSAK*	38	lamin A
11	1025.18	1023.11	0.20%	NIYSEELR	39	lamin A
12	805.83	803.91	0.24%	TALSEKR	40	lamin A
17	1349.52	1347.56	0.15%	<u>LALDMEIHAYR</u> **	41	lamin A
17	1009.78	1009.18	0.06%	EMAEMRAR	42	lamin A
21	1912.74	1913.07	0.02%	EELDFQKNIYSEELR*	43	lamin A
22	1896.58	1894.13	0.13%	<u>MQQQLDEYQELLDIK</u> **	44	lamin A
22	1913.03	1913.07	0.00%	EELDFQKNIYSEELR*	45	lamin A

\* Denotes a peptide that appeared in two adjacent HPLC fractions

\*\* Underlining reflects sequences confirmed by Edman degradation

Cervical cancer-associated proteins may be identified using well-known techniques based upon the partial amino acid sequences provided above. Thus, the cervical cancer-associated proteins detected according to methods of the invention may be referred to as comprising a continuous sequence shown in the above-noted sequence fragments. It is appreciated that the skilled artisan, in view of the foregoing disclosure, would be able to produce an antibody directed against any cervical cancer-associated protein identified by the methods described herein. Moreover, the skilled artisan, in view of the foregoing disclosure, would be able to produce nucleic acid sequences which encode the fragments described above, as well as nucleic acid sequences complementary thereto. In addition, the skilled artisan using conventional recombinant DNA methodologies, for example, by screening a cDNA library with such a nucleic acid sequence, would be able to isolate full length nucleic acid sequences encoding target

cervical cancer-associated proteins. Such full length nucleic acid sequences, or fragments thereof, may be used to generate nucleic acid-based detection systems or therapeutics.

#### *Example 4*

##### *Production of Antibodies Which Bind Specifically to Cervical Cancer-associated Proteins.*

Once identified, a cervical cancer-associated protein, such as a CvC-1 through CvC-5, may be detected in a tissue or body fluid sample using numerous binding assays that are well known to those of ordinary skill in the art. For example, as discussed above, a cervical cancer-associated protein may be detected in either a tissue or body fluid sample using an antibody, for example, a monoclonal antibody, which bind specifically to an epitope disposed upon the cervical cancer-associated protein. In such detection systems, the antibody preferably is labeled with a detectable moiety.

Provided below is an exemplary protocol for the production of an anti-cervical cancer-associated monoclonal antibody. Other protocols also are envisioned. Accordingly, the particular method of producing antibodies to target proteins is not envisioned to be an aspect of the invention.

Balb/c by J mice (Jackson Laboratory, Bar Harbor, ME) are injected intraperitoneally with the target protein, e.g., CvC-3 protein isolated from HeLa cell nuclear matrix, every 2 weeks until the immunized mice obtain the appropriate serum titer. Thereafter, the mice are injected with 3 consecutive intravenous boosts. Freund's complete adjuvant (Gibco, Grand Island) is used in the first injection, incomplete Freund's in the second injection; and saline is used for subsequent intravenous injections. The animal is then sacrificed and its spleen removed. Spleen cells (or lymph node cells) then are fused with a mouse myeloma line, e.g., using the method of Kohler *et al.* (1975) *Nature* 256: 495, the disclosure of which is incorporated herein by reference. Hybridomas producing antibodies that react with the target proteins then are cloned and grown as ascites. Hybridomas are screened by nuclear reactivity against the cell line that is the source of

the immunogen, and by tissue immunohistochemistry using standard procedures known in the immunology art. Detailed descriptions of screening protocols, ascites production and immunoassays also are disclosed in PCT/US92/09220 published May 13, 1993, the disclosure of which is incorporated herein by reference.

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### *Example 5*

#### *Antibody-based Assay for Detecting Cervical Cancer in an Individual*

The following assay has been developed for tissue samples, however, it is contemplated that similar assays for testing fluid samples may be developed without undue experimentation. A typical assay may employ a commercial immunodetection kit, for example, the ABC Elite Kit from Vector Laboratories, Inc.

A biopsy sample, for example, a Pap smear is removed from the patient under investigation in accordance with the appropriate medical guidelines. The sample then is applied to a glass microscope slide and the sample fixed in cold acetone for 10 minutes. Then, the slide is rinsed in distilled water and pretreated with a hydrogen peroxide containing solution (2 mL 30% H<sub>2</sub>O<sub>2</sub> and 30 mL cold methanol). The slide is then rinsed in a Buffer A comprising Tris Buffered Saline (TBS) with 0.1% Tween and 0.1% Brij. A mouse anti-cervical cancer-associated protein monoclonal antibody in Buffer A is added to the slide and the slide then is incubated for one hour at room temperature. The slide is then washed with Buffer A, and a secondary antibody (ABC Elite Kit, Vector Labs, Inc) in Buffer A is added to the slide. The slide is then incubated for 15 minutes at 37°C in a humidity chamber. The slides are washed again with Buffer A, and the ABC reagent (ABC Elite Kit, Vector Labs, Inc.) is then added to the slide for amplification of the signal. The slide is then incubated for a further 15 minutes at 37°C in the humidity chamber.

The slide then is washed in distilled water, and a diaminobenzenedine (DAB) substrate added to the slide for 4-5 minutes. The slide is then rinsed with distilled water, counterstained

with hematoxylin, rinsed with 95% ethanol, rinsed with 100% ethanol, and then rinsed with xylene. A cover slip is then applied to the slide and the result observed by light microscopy.

**Equivalents.**

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The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and  
10 all changes that come within the meaning and range of equivalency of the claims are intended to be embraced therein.

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